pUB6/V5-His A, B, and C

Catalog no. V250-20

Version B

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General Information

Contents

20 µg each pUB6/V5-His A, B, and C, lyophilized in TE, pH 8.0
20 µg pUB6/V5-His/*lac*Z, lyophilized in TE, pH 8.0

Shipping/Storage

Lyophilized plasmids are shipped at room temperature and stored at -20°C.

Product Qualification

pUB6/V5-His A, B, and C and pUB6/V5-His/*lac*Z are qualified by restriction digest. The table below lists the restriction enzymes and the expected fragments.

Vector	Restriction Enzyme	Expected Fragment
pUB6/V5-His A	*Apa* I	5462 bp
	Sac II	5462 bp
	*Bst*E II	No cut
	Stu I	3684 bp, 1778 bp
pUB6/V5-His B	*Apa* I	5466 bp
	Sac II	4193 bp, 1273 bp
	*Bst*E II	No cut
	Stu I	3684 bp, 1782 bp
pUB6/V5-His C	*Apa* I	No cut
	Sac II	5462 bp
	*Bst*E II	5462 bp
	Stu I	3688 bp, 1774 bp
pUB6/V5-His/*lac*Z	*Apa* I	No cut
	Sac II	8509 bp
	*Bst*E II	8509 bp
	Stu I	4825 bp, 3684 bp

Methods

Overview

Introduction

pUB6/V5-His A, B, and C are 5.5 kb vectors designed for overproduction of recombinant proteins in mammalian cell lines. Features of the vectors allow purification and detection of expressed proteins (see pages 15-16 for more information). High-level stable and transient expression can be carried out in most mammalian cells. The vectors contain the following elements:

- Human ubiquitin C promoter (hUbC) for high-level expression across a broad range of species and cell types (Schorpp *et al.*, 1996; Wulff *et al.*, 1990) (see page 14 for more information).
- Three reading frames to facilitate in-frame cloning with a C-terminal peptide encoding the V5 epitope and a polyhistidine (6xHis) metal-binding tag.
- Blasticidin resistance gene (*bsd*) for selection of stable cell lines (see page 11 for more information).
- Episomal replication in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS-1, COS-7).

The control plasmid, pUB6/V5-His/*lacZ*, is included for use as a positive control for transfection, expression, and detection in the cell line of choice.

Experimental Outline

Use the following outline to clone and express your gene of interest in pUB6/V5-His.

1. Consult the multiple cloning sites described on pages 4-6 to determine which vector (A, B, or C) should be used to clone your gene in frame with the C-terminal V5 epitope and polyhistidine tag.
2. Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on 50 to 100 µg/ml ampicillin (or 50 µg/ml blasticidin).
3. Analyze your transformants for the presence of insert by restriction digestion.
4. Select a transformant with the correct restriction pattern and use sequencing to confirm that your gene is cloned in frame with the C-terminal peptide.
5. Transfect your construct into the cell line of choice. Generate a stable cell line, if desired.
6. Test for expression of your recombinant gene by western blot analysis or other functional assay. For antibodies to the V5 epitope or the polyhistidine, C-terminal tag, see the next page.
7. To purify your recombinant protein, you may use metal-chelating resin such as ProBond™. ProBond™ resin is available separately (see next page for ordering information).

Accessory Products

Introduction

The products listed below are designed to help you detect and purify your recombinant fusion protein expressed from pUB6/V5-His. In addition, we have a wide variety of mammalian expression vectors, many of which can be utilized with pUB6/V5-His to express and detect multiple proteins in the same cell (see below).

Antibodies for Detection

If you do not have an antibody to your protein, Invitrogen offers the Anti-V5 antibodies or the Anti-His(C-term) antibodies to detect your recombinant fusion protein. Horseradish peroxidase(HRP)-conjugated antibodies are available for convenient one-step detection.

Antibody	Epitope	Catalog no.
Anti-V5	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991): GKPIPNPLLGLDST	R960-25
Anti-V5-HRP	same as above	R961-25
Anti-His(C-term)	Detects the C-terminal polyhistidine tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP	same as above	R931-25

ProBond™ Resin

Ordering information for ProBond™ resin is provided below.

Item	Amount	Catalog no.
ProBond™ Purification System	12 ml precharged ProBond™ resin columns, and buffers for native and denaturing purification	K850-01
ProBond™ Purification Kit with Anti-V5-HRP Antibody	1 Kit	K854-01
ProBond™ Resin	50 ml	R801-01
	150 ml	R801-15

Expression Vectors

We have a wide variety of mammalian expression vectors utilizing the CMV or EF-1α promoters. Vectors are available with the Xpress™ (N-terminal), *c-myc* (C-terminal), or V5 (C-terminal) epitopes for detection and either the neomycin, blasticidin, or Zeocin™ resistance genes. All vectors utilize the polyhistidine tag for purification. For more information on the mammalian expression vectors available, see our web site (www.invitrogen.com) or call Technical Service (page 18).

Cloning into pUB6/V5-His A, B, and C

Introduction

Diagrams are provided on pages 4-6 to help you ligate your gene of interest in frame with the coding sequence of the C-terminal peptide. General considerations for transformation are listed below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) (See **References**, page 21).

E. coli Strain for Transformation

Many *E. coli* strains are suitable for the propagation of this vector, including TOP10F′ (Catalog no. C615-00), JM109, and INVαF′ (Catalog no. C658-00). We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10F′ is available as chemically competent or electrocompetent cells from Invitrogen.

Item	Quantity	Catalog no.
Electrocomp™ TOP10F′	5 x 80 µl	C665-55
Ultracomp™ TOP10F′ (chemically competent cells)	5 x 300 µl	C665-03
One Shot™ TOP10F′ (chemically competent cells)	21 x 50 µl	C3030-03

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of pUB6/V5-His

To propagate and maintain the pUB6/V5-His vectors, resuspend each vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.

Use this stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10F′, DH5α, JM109, or equivalent. Select transformants on LB plates containing 50 to 100 µg/ml ampicillin (or 50 µg/ml blasticidin). Be sure to prepare a glycerol stock of plasmid-containing *E. coli* strain for long-term storage (see page 7).

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Cloning into pUB6/V5-His A, B, and C, continued

Cloning Considerations

Your insert should contain a Kozak translation initiation sequence for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Note that other sequences are possible (see references above), but the A at position -3 and the G at position +4 are the most critical (shown in bold). The ATG initiation codon is shown underlined.

ANNATGG

To express your gene as a recombinant fusion protein, you must clone your gene in frame with the C-terminal peptide. The vector is supplied in three reading frames to facilitate cloning. See below and pages 5-6 to develop a cloning strategy.

If you wish to express your protein WITHOUT the C-terminal peptide, be sure to include a stop codon.

Multiple Cloning Site of pUB6/V5-His A

Below is the multiple cloning site for pUB6/V5-His A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. **Note that there is a stop codon between the *Bam*H I site and the *Bst*X I site.** The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence may be downloaded from our web site (www.invitrogen.com)** or from Technical Service (see page 18). For more information on the hUbC promoter, see page 14.

```
                                                          UB forward priming site
1121  TTTAGGCACC TTTTGAAATG TAATCATTTG GGTCAATATG TAATTTTCAG TGTTAGACTA GTAAATTGTC CGCTAAATTC

                                    Hind III    Asp718 I  Kpn I      Sac I BamH I                BstX I*
1201  TGGCCGTTTT TGGCTTTTTT GTTAGAC GAA GCT TGG TAC CGA GCT CGG ATC CAC TAG TCC AGT GTG GTG
                                      Glu Ala Trp Tyr Arg Ala Arg Ile His *** Ser Ser Val Val

      EcoR I        Pst I  EcoR V          BstX I*   Not I     Xho I     Xba I          Apa I  BstB I
1270  GAA TTC TGC AGA TAT CCA GCA CAG TGG CGG CCG CTC GAG TCT AGA GGG CCC TTC GAA GGT AAG CCT
      Glu Phe Cys Arg Tyr Pro Ala Gln Trp Arg Pro Leu Glu Ser Arg Gly Pro Phe Glu Gly Lys Pro

                          V5 epitope                              Age I      Polyhistidine region
1336  ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA GT
      Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His His ***

      Pme I                pcDNA3.1/BGH reverse priming site
1401  TTAAACCCGC TGATCAGCCT CGACTGTGCC TTCTAGTTGC CAGCCATCTG TTGTTTGCCC CTCCCCCGTG CCTTCCTTGA

                                        BGH polyadenylation signal
1481  CCCTGGAAGG TGCCACTCCC ACTGTCCTTT CCTAATAAAA TGAGGAAATT GCATCGCATT GTCTGAGTAG GTGTCATTCT
```

*Note that there are two *Bst*X I sites in the polylinker.

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Cloning into pUB6/V5-His A, B, and C, continued

Multiple Cloning Site of pUB6/V5-His B

Below is the multiple cloning site for pUB6/V5-His B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence may be downloaded from our web site (www.invitrogen.com)** or from Technical Service (see page 18). For more information on the hUbC promoter, see page 14.

```
                                                                    UB forward priming site
1121  TTTAGGCACC TTTTGAAATG TAATCATTTG GGTCAATATG TAATTTTCAG TGTTAGACTA GTAAATTGTC CGCTAAATTC

                                        HindIII     Asp718I  KpnI       SacI BamHI                          BstXI*  EcoRI
1201  TGGCCGTTTT TGGCTTTTTT GTTAGACG AAG CTT GGT ACC GAG CTC GGA TCC ACT AGT CCA GTG TGG TGG
                                      Lys Leu Gly Thr Glu Leu Gly Ser Thr Ser Pro Val Trp Trp

                 PstI  EcoRV          BstXI*   NotI       XhoI      XbaI              ApaI       BstBI
1271  AAT TCT GCA GAT ATC CAG CAC AGT GGC GGC CGC TCG AGT CTA GAG GGC CCG CGG TTC GAA GGT AAG
      Asn Ser Ala Asp Ile Gln His Ser Gly Gly Arg Ser Ser Leu Glu Gly Pro Arg Phe Glu Gly Lys

                                  V5 epitope                                    AgeI          Polyhistidine region
1337  CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA
      Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His His ***

      PmeI                  pcDNA3.1/BGH reverse priming site
1403  GTTTAAAC CCGCTGATCA GCCTCGACTG TGCCTTCTAG TTGCCAGCCA TCTGTTGTTT GCCCCTCCCC CGTGCCTTCC

                                                BGH polyadenylation signal
1481  TTGACCCTGG AAGGTGCCAC TCCCACTGTC CTTTCCTAAT AAAATGAGGA AATTGCATCG CATTGTCTGA GTAGGTGTCA
```

*Note that there are two *Bst*X I sites in the polylinker.

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Cloning into pUB6/V5-His A, B, and C, continued

Multiple Cloning Site of pUB6/V5-His C

Below is the multiple cloning site for pUB6/V5-His C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence may be downloaded from our web site (www.invitrogen.com)** or from Technical Service (see page 18). For more information on the hUbC promoter, see page 14.

UB forward priming site

```
1121  TTTAGGCACC TTTTGAAATG TAATCATTTG GGTCAATATG TAATTTTCAG TGTTAGACTA GTAAATTGTC CGCTAAATTC

                                        Hind III   Asp718 I  Kpn I     Sac I  BamH I                    BstX I*  EcoR I
1201  TGGCCGTTTT TGGCTTTTTT GTTAGACGA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTC CAG TGT GGT GGA
                                      Ser Leu Val Pro Ser Ser Asp Pro Leu Val Gln Cys Gly Gly

              Pst I  EcoR V            BstX I*  Not I      Xho I    BstE II          BstB I          V5 epitope
1272  ATT CTG CAG ATA TCC AGC ACA GTG GCG GCC GCT CGA GGT CAC CCA TTC GAA GGT AAG CCT ATC CCT
      Ile Leu Gln Ile Ser Ser Thr Val Ala Ala Ala Arg Gly His Pro Phe Glu Gly Lys Pro Ile Pro

                                                  Age I                Polyhistidine region          Pme I
1338  AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT CAT CAT CAC CAT CAC CAT TGA GTTTAA
      Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly His His His His His His ***

                  pcDNA3.1/BGH Reverse priming site
1401  ACCCGCTGAT CAGCCTCGAC TGTGCCTTCT AGTTGCCAGC CATCTGTTGT TTGCCCCTCC CCCGTGCCTT CCTTGACCCT

                                    BGH polyadenylation signal
1481  GGAAGGTGCC ACTCCCACTG TCCTTTCCTA ATAAAATGAG GAAATTGCAT CGCATTGTCT GAGTAGGTGT CATTCTATTC
```

*Note that there are two *BstX* I sites in the polylinker.

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Cloning into pUB6/V5-His A, B, and C, continued

Transformation of Ligation Mixtures

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10F′, DH5α) and select on LB plates containing 50-100 µg/ml ampicillin or 50 µg/ml blasticidin. Select 10-20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct with the UB Forward and pcDNA3.1/ BGH Reverse primer sequences to confirm that your gene is fused in frame with the V5 epitope and the C-terminal polyhistidine tag. Refer to the diagram on pages 4-6 for the sequence and location of the primer binding sites.

For your convenience, Invitrogen offers a custom primer synthesis service. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 18).

Preparing a Glycerol Stock

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at -20°C.

- Streak the original colony out for single colonies on an LB plate containing 50 µg/ml ampicillin (or 50 µg/ml blasticidin). Incubate the plate at 37°C overnight.
- Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml ampicillin (or 50 µg/ml blasticidin).
- Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
- Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
- Store at -80°C.

Transfection and Analysis

Introduction

Once you have confirmed that your construct is in the correct orientation and fused to the C-terminal peptide (if desired), then you are ready to transfect your cell line of choice. We recommend that you include the positive control vector and a mock transfection to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells and salt will interfere with lipids, decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg, Catalog no. K1910-01), or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (e.g. HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. It is recommended that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (**Reference** section, page 20).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1987; Felgner *et al.*, 1989), and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Invitrogen offers the Calcium Phosphate Transfection Kit (K2780-01) and a large variety of transfection reagents for mammalian transfection. For more information on the reagents available, see our Web site (www.invitrogen.com) or call Technical Service (see page 18).

Positive Control

pUB6/V5-His/*lacZ* is provided as a positive control vector for mammalian transfection and expression (see page 17). It may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the control of the human ubiquitin C (hUbC) promoter. A successful transfection will result in β-galactosidase expression that can be easily assayed (see next page).

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Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Invitrogen offers the β-Gal Assay Kit (Catalog no. K1455-01) and the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

Detection of Fusion Proteins

Several antibodies are available from Invitrogen that can be used to detect expression of your fusion protein from pUB6/V5-His (see page 2).

To detect the fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. We recommend that you perform a time course to optimize expression of the fusion protein (*e.g.* 24, 48, 72 hours, etc. after transfection). To lyse cells:

1. Wash cell monolayers (~10^6 cells) once with phosphate-buffered saline (PBS).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (see recipe below). Other lysis buffers are suitable.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.
5. Vortex the cell lysate and centrifuge at 10,000 x g for 10 minutes to pellet nuclei. Transfer the supernatant to a fresh tube. Assay the supernatant for protein concentration. **Note**: Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 ml, combine:

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

Note: Protease inhibitors may be added at the following concentrations:

1 mM PMSF
1 µg/ml Pepstatin
1 µg/ml Leupeptin

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Transfection and Analysis, continued

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine region will add approximately 5 kDa to the size of your protein.

Purification

You will need 5 x 10^6 to 1 x 10^7 **transfected** cells for purification of your protein on a 2 ml ProBond™ column (or other metal-chelating column). Refer to the manufacturer's instructions before attempting to purify your fusion protein. To prepare cells for lysis, refer to the protocol on page 13.

Creation of Stable Cell Lines

Introduction

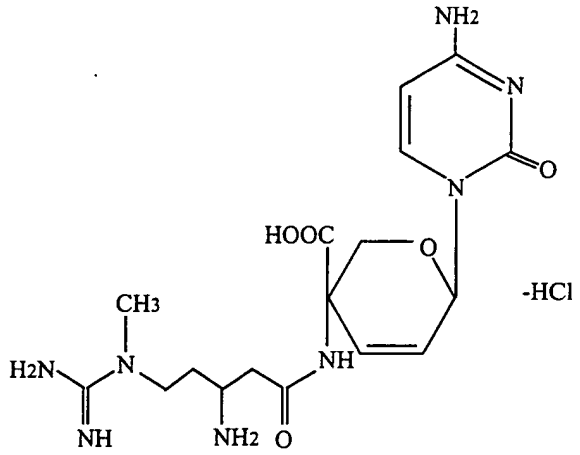
The pUB6/V5-His vectors contain the blasticidin resistance gene (*bsd*) to allow for selection of stable cell lines using blasticidin (Kimura *et al.*, 1994). We recommend that you test the sensitivity of your mammalian host cell to blasticidin as natural resistance varies among cell lines. General information and guidelines are provided below for your convenience.

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *bsd from Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular Weight, Formula, and Structure

The formula for blasticidin is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (e.g. a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

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Creation of Stable Cell Lines, continued

Preparing and Storing Stock Solutions

Blasticidin may be obtained from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Water is generally used to prepare stock solutions of 5 to 10 mg/ml.

- Dissolve blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see last point below) and freeze at -20°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1-2 weeks at +4°C and 6-8 weeks at -20°C.
- pH of the aqueous solution should not exceed 7 to prevent inactivation of blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and discard the unused portion.

Possible Sites for Linearization

To obtain stable transfectants, you may choose to linearize your vector before transfection. While linearizing your vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts the gene of interest. The table below lists some unique sites that may be used to linearize your construct prior to transformation. **Other restriction sites are possible.** Note that for the enzymes listed below, the cleavage site is indicated for versions A, B, and C of pUB6/V5-His. Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Location	Supplier
Bgl II	Upstream of hUbC promoter	Invitrogen (catalog no. 15213-010)
*Bst*1107 I	End of SV40 polyA	AGS*, Fermentas, Takara
Sap I	Backbone	New England Biolabs
*Bsp*LU 11I	Backbone	Boehringer-Mannheim
*Alw*N I	pMB1 origin	Amersham, New England Biolabs, Life Technologies
*Eam*1105 I	Ampicillin gene	AGS*, Fermentas, Takara
Bgl I	Ampicillin gene	Many
Fsp I	Ampicillin gene	Many
Sca I	Ampicillin gene	Invitrogen (catalog no. 15436-017)
Ssp I	Backbone	Invitrogen (catalog no. 15458-011)

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Creation of Stable Cell Lines, continued

Selection in Mammalian Cell Lines

To generate a stable cell line expressing your protein, you need to determine the minimum concentration of blasticidin required to kill your untransfected host cell line. Typically, concentrations between 2 and 10 µg/ml blasticidin are sufficient to kill the untransfected host cell line. Test a range of concentrations (see below) to ensure that you determine the minimum concentration necessary for your cell line.

- Seed cells at 20-25% confluency for each time point (~6 time points) and allow the cells to adhere overnight.
- The next day, substitute culture medium with medium containing varying concentrations of blasticidin (e.g. 0, 1, 3, 5, 7.5, and 10 µg/ml blasticidin).
- Replenish the selective medium every 3-4 days. Cells sensitive to blasticidin will round up and detach from the plate. Dead cells will accumulate in the medium.
- Count the number of viable cells at regular intervals to determine the appropriate concentration of blasticidin that prevents growth.

Selection of Stable Integrants

Once the appropriate concentration of blasticidin is determined, you can generate a stable cell line with your construct.

- Transfect your cells using the optimal protocol for your cell line. Include a sample of untransfected cells as a negative control.
- 48 hours after transfection, split the cells into fresh medium containing blasticidin at the appropriate concentration for your cell line. Split the cells such that they are no more than 25% confluent.
- Replenish selective medium every 3-4 days until blasticidin-resistant colonies are detected.
- Pick and expand colonies.

Preparation of Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purification of your protein on ProBond™. You will need 5 x 10^6 to 1 x 10^7 cells for purification of your protein on a 2 ml ProBond™ column (see Xpress™ Protein Purification manual).

1. Seed cells (from a stable cell line) in either five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are 80-90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium (if necessary) and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 1500 x g for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 1500 x g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -70°C until needed.

Lysis of Cells

If you are using ProBond™ resin, refer to the ProBond™ Protein Purification manual for details about sample preparation for chromatography.

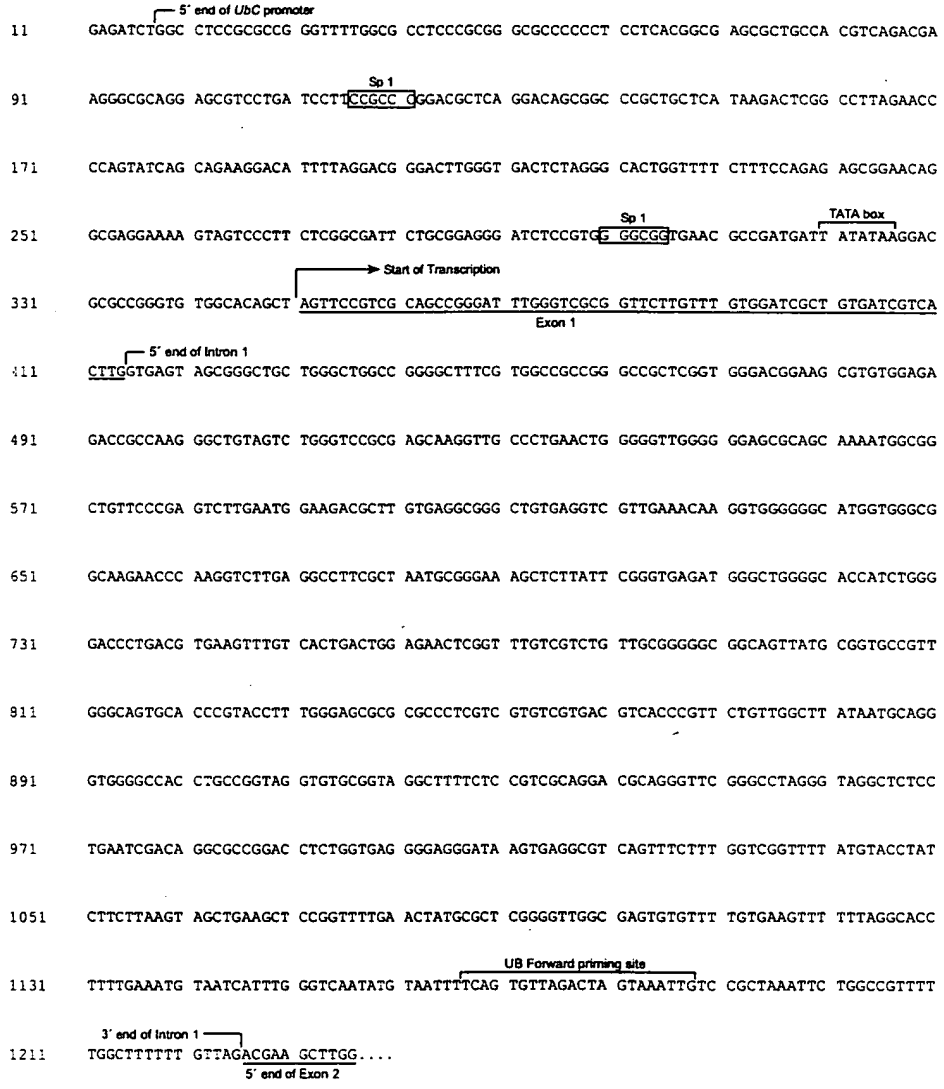
If you are using other metal-chelating resin, refer to the manufacturer's instruction for recommendations on sample preparation.

Appendix

Human UbC Promoter

Description

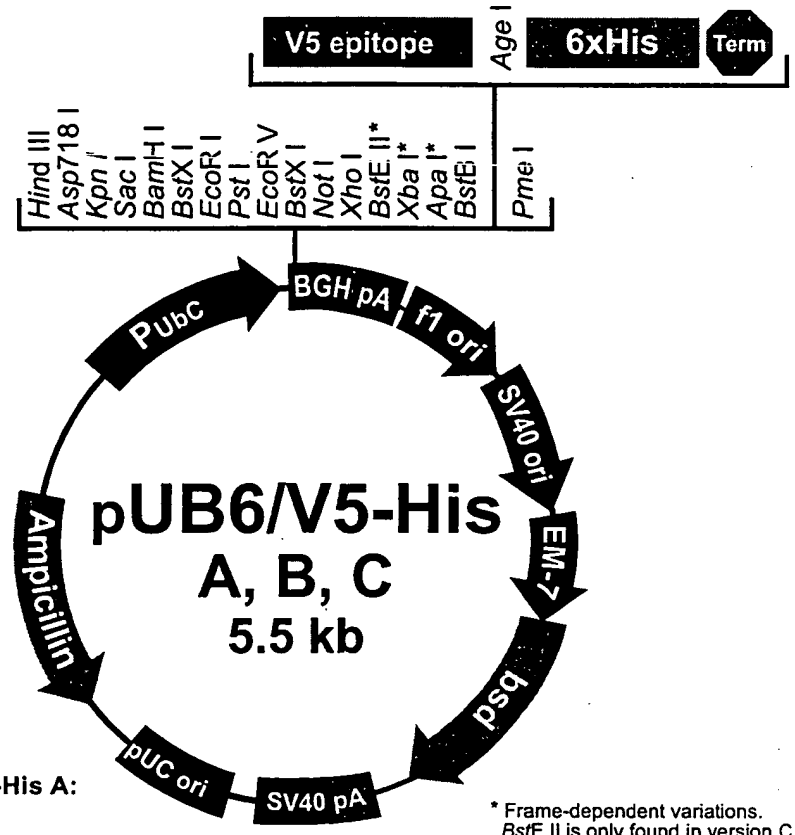
The human UbC promoter allows high-level expression of recombinant protein in most mammalian cell lines (Wulff *et al.*, 1990) and in virtually all tissues tested in transgenic mice (Schorpp *et al.*, 1996). The diagram below shows the features of the UbC promoter used in pUB6/V5-His (Nenoi *et al.*, 1996). Features are marked as per Nenoi, *et al.*, 1996.



pUB6/V5-His Vector

Map of pUB6/V5-His

The figure below summarizes the features of the pUB6/V5-His vectors. **The sequences for pUB6/V5-His A, B, and C are available for downloading from our Web site (http://www.invitrogen.com) or from Technical Service (see page 18).**



* Frame-dependent variations. *Bst*E II is only found in version C. In addition, there are no *Xba* I or *Apa* I sites in version C.

Comments for pUB6/V5-His A:
5463 nucleotides

UbC promoter: bases 18-1227
UB forward priming site: bases 1167-1188
Multiple cloning site: bases 1229-1326
V5 epitope: bases 1327-1368
Polyhistidine tag: bases 1378-1395
pcDNA3.1/BGH reverse priming site: bases 1418-1435
BGH polyadenylation signal: bases 1421-1648
f1 origin: bases 1694-2122
SV40 promoter and origin: bases 2149-2458
EM-7 promoter: bases 2506-2561
Blasticidin resistance gene: bases 2580-2978
SV40 polyadenylation signal: bases 3136-3266
pUC origin: bases 3649-4322
Ampicillin resistance gene: bases 4467-5327

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pUB6/V5-His Vector, continued

Features of pUB6/V5-His

pUB6/V5-His A (5463 bp), pUB6/V5-His B (5467 bp), and pUB6/V5-His C (5459 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
Human ubiquitin C (hUbC) promoter	Permits overexpression of your recombinant protein in a broad range of mammalian cell types (Hershko and Ciechanover, 1982; Wulff *et al.*, 1990; Schorpp *et al.*, 1996)
Multiple cloning site in three reading frames	Allows insertion of your gene and facilitates cloning in frame with the V5 epitope and the C-terminal polyhistidine tag
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of your recombinant protein with the Anti-V5 Antibody (Catalog no. R960-25) or Anti-V5-HRP Antibody (Catalog no. R961-25) (Southern *et al.*, 1991)
C-terminal polyhistidine tag	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™ (R801-01) In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody (Catalog no. R930-25) and the Anti-His (C-term)-HRP Antibody (Catalog no. R931-25) (Lindner *et al.*, 1997)
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM-7 promoter	For expression of the blasticidin resistance gene in *E. coli.*
Blasticidin resistance gene (*bsd*)	Selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994)
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*

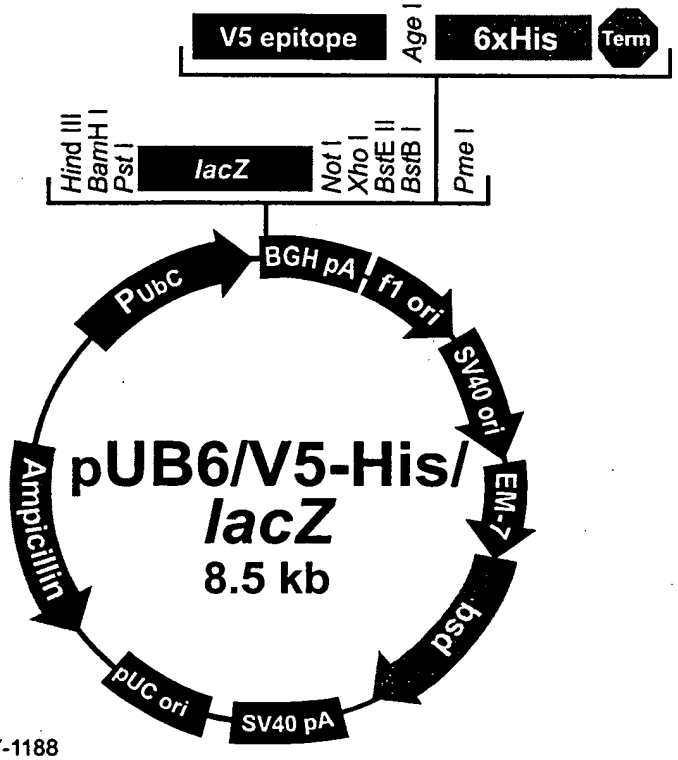
pUB6/V5-His/*lacZ*

Description

pUB6/V5-His/*lacZ* is a 8510 bp control vector containing the gene for β-galactosidase. This vector was constructed by ligating a 3190 bp *Hind* III-*Age* I fragment containing the *lacZ* gene and the V5 epitope to a 5320 bp *Hind* III-*Age* I fragment containing the hUbC promoter, polyhistidine tag and blasticidin resistance gene from pUB6/V5-His A.

Map of Control Vector

The figure below summarizes the features of the pUB6/V5-His/*lacZ* vector. **The complete nucleotide sequence for pUB6/V5-His/*lacZ* is available for downloading from ourWeb site (http://www.invitrogen.com) or by contacting Technical Service.** (see next page).



Comments for pUB6/V5-His/*lacZ*:
8510 nucleotides

UbC promoter: bases 18-1227
UB forward priming site: bases 1167-1188
LacZ ORF: bases 1290-4346
V5 epitope: bases 4374-4415
Polyhistidine tag: bases 4425-4442
pcDNA3.1/BGH reverse priming site: bases 4465-4482
BGH polyadenylation signal: bases 4468-4695
f1 origin: bases 4741-5169
SV40 promoter and origin: bases 5196-5505
EM-7 promoter: bases 5553-5608
Blasticidin resistance gene: bases 5627-6025
SV40 polyadenylation signal: bases 6183-6313
pUC origin: bases 6696-7369
Ampicillin resistance gene: bases 7514-8374

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

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- Obtain citations for Invitrogen products
- Request catalog and product literature

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Technical Service, continued

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Limited Use Label License No. 51: Blasticidin and the Blasticidin Selection Marker

Blasticidin and the blasticidin resistance gene (*bsd*) are sold under patent license and may be used for **research purposes only**. Inquiries for commercial use should be directed to: Kaken Pharmaceutical Company, Ltd., Bunkyo Green Court, Center Office Building, 19-20 Fl, 28-8 Honkomagome 2-chome, Bunkyo-ku, Tokyo 113-8650, Japan.
Tel: 81 3-5977-5008; Fax: 81 3-5977-5008.

Product User Registration Card

Please complete and return the enclosed Product User Registration Card for pUB6/V5-His A, B, and C that you purchase. This will serve as a record of your purchase and will allow Invitrogen to provide you with critical product updates. The agreement outlined above becomes effective upon our receipt of your Product User Registration Card or 10 days following the sale of pUB6/V5-His A, B, and C to you. Use of the vectors at any time results in immediate obligation to the terms and conditions stated in this Agreement.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Mol. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nuc. Acids Res. *15*, 1311-1326.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987). Lipofectin: A Highly Efficient, Lipid-mediated DNA-transfection Procedure. Proc. Natl. Acad. Sci. USA *84*, 7413-7417.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Goodwin, E. C., and Rottman, F. M. (1992). The 3′-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation. J. Biol. Chem. *267*, 16330-16334.

Hershko, A., and Ciechanover, A. (1982). Mechanisms of Intracellular Protein Breakdown. Ann. Rev. Biochem. *51*, 335-364.

Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991). Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. Exp. Cell Res. *197*, 229-233.

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994). Blasticidin S Deaminase Gene from *Aspergillus terreus* (*BSD*): A New Drug Resistance Gene for Transfection of Mammalian Cells. Biochim. Biophys. Acta *1219*, 653-659.

Kozak, M. (1987). An Analysis of 5′-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nuc. Acids Res. *15*, 8125-8148.

Kozak, M. (1991). An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biol. *115*, 887-903.

Kozak, M. (1990). Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305.

Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Pluckthun, A. (1997). Specific Detection of His-tagged Proteins With Recombinant Anti-His Tag scFv-Phosphatase or scFv-Phage Fusions. BioTechniques *22*, 140-149.

Miller, J. H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Nenoi, M., Mita, K., Ichimura, S., Cartwright, I. L., Takahashi, E., Yamaguchi, M., and Tsuji, H. (1996). Heterogeneous Structure of the Polyubiquitin Gene UbC of HeLa S3 Cells. Gene *175*, 179-185.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

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References, continued

Schorpp, M., Jäger, R., Schellander, K., Schenkel, J., Wagner, E. F., Weiher, H., and Angel, P. (1996). The Human Ubiquitin C Promoter Directs High Ubiquitous Expression of Transgenes in Mice. Nuc. Acids Res. *24*, 1787-1788.

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557.

Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958). Blasticidin S, A New Antibiotic. The Journal of Antibiotics, Series A *11*, 1-5.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

Wulff, B. S., O'Hare, M. M., Boel, E., Theill, L. E., and Schwartz, T. W. (1990). Partial Processing of the Neuropeptide Y Precursor in Transfected CHO Cells. FEBS Lett. *261*, 101-105.

Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965). Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. J. Biochem. (Tokyo) *57*, 667-677.